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REMARKS

Interview Summary

The Examiner included with the Office Action dated 21 October 2003 an "Interview Summary" regarding an "interview" occurring by telephone on 15 October 2003. This paper noted the telephone call from Examiner Kam to Applicants' attorney Carol A. Egner, in which Examiner Kam extended the attorney the courtesy of informing her that prosecution of the application was being reopened. No matter of substance affecting the claims was discussed at that time.

Amendments to Claims

Claims 12, 13, 17 and 19 have been amended. Support for the amendments to Claims 12 and 13 is found on page 12, line 25 to page 13, line 7, wherein COMP is described as being cleaved into fragments by trypsin (the fragments visualized by polyacrylamide gel electrophoresis as bands), the size of the fragments depending on the calcium concentration present in the trypsin digestion. Claims 17 and 19 have been amended to spell out "ELISA" as "enzyme-linked immunosorbent assay," as it is known in the art.

Rejection of Claims 12, 13, 17, 19, 39 and 42 Under 35 U.S.C. § 112, Second Paragraph

Claims 12, 13, 17, 19, 39 and 42 have been rejected under 35 U.S.C. § 112, second paragraph, as they are said to be indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 12 and 13 are said to be indefinite as to how different bands are produced when the claims merely indicate human cartilage oligomeric matrix protein is cleaved by trypsin. Claims 12 and 13 have been amended to make clear that the protein is in a conformation which can be cleaved by trypsin into fragments of the sizes specified in the claims.

Claims 17 and 19 are said to be indefinite because of the use of the term "ELISA." It is well known in the art that the term "ELISA" stands for "enzyme-linked immunosorbent assay," and the latter term has been substituted for the former term in Claims 17 and 19.

Claims 17 and 19 are also said to be indefinite "as to what else are included in the kit besides human cartilage oligomeric matrix protein since the term 'comprising' is cited in the claim." Applicants are unaware of how the term "comprising" makes a claim indefinite. One component of the kit is specified, providing a definite meaning to the claim. The Examiner is requested to point out patent rules or case law to clarify the rejection.

Claim 39 is said to be indefinite because the use of the term "a differentiation agent" is said to render the claim indefinite. One of ordinary skill in the art would understand that the term "differentiation agent" means a substance that induces cells to change from the undifferentiated state to the differentiated state. Examples of differentiation agents are given in the specification at page 5, lines 15-19. It is not necessary to explain in a composition of matter claim the purpose or function of any of the components of the composition. It should be clear enough from the specification (for example, page 5, lines 9-27) that the composition of Claim 39 can be used, for instance, as a substrate to culture cells normally present in cartilage, and that the resulting composition can be implanted at sites in the body to substitute for, or to augment, cartilage.

Claim 42 is said to be indefinite "as to whether collagens include type I collagen gel or type II collagen gel since the claim recites the matrix comprises type I collagen gel or type II collagen gel and further comprises collagens."

"Collagens" do not include type I collagen gel or type II collagen gel. Collagen gel is prepared by methods well known in the art, and requires certain conditions such as concentration of purified collagen (extracted from a source such as animal tendon, and kept in an aqueous solution), temperature and pH to form a gel or matrix. See Toung, J.S. *et al., Arch. Otolaryngol. Head Neck Surg. 125*:451-455 (April, 1999), especially fourth paragraph of Materials and Methods on page 452; copy enclosed as Exhibit A. About 15 types of collagen have been identified so far. See pages 1096-1098 of *Molecular Biology of the Cell*, 4th edition, B. Alberts et al., eds., Garland Science, New York, NY, 2002; copy enclosed as Exhibit B. A type I collagen gel can include, for example, a different type of collagen that may or may not associate with type I collagen to form part of the gel.

Rejection of Claims 12 and 13 Under 35 U.S.C. § 102(b)

Claims 12 and 13 are rejected under 35 U.S.C. § 102(b) as they are said to be anticipated by Newton *et al., Genomics 24*:435-439 (1994).

Newton *et al.* describe the cloning and sequencing of a gene encoding human cartilage matrix oligomeric protein. From the nucleotide sequence of the cDNA, the amino acid sequence can be deduced. Newton *et al.* do not report any scheme to purify human cartilage matrix oligomeric protein, nor do they describe the purified protein.

As was demonstrated in the specification, the property of which products are produced by trypsin digestion of human cartilage matrix oligomeric protein is not an inherent property of the protein, unlike the amino acid sequence or composition. Which products are produced by trypsin digestion of human cartilage matrix oligomeric protein varies according to the conformation of the protein, which, as demonstrated in the specification, differs according to the calcium concentration. Human cartilage matrix oligomeric protein in the conformation described in Claims 12 and 13 was not disclosed in Newton *et al.* Nothing in Newton *et al.* would lead one of ordinary skill in the art to believe that the conformation of human cartilage matrix oligomeric protein described in Claims 12 and 13 could exist.

Rejection of Claim 42 Under 35 U.S.C. § 102(a)

Claim 42 has been rejected under 35 U.S.C. § 102(a) as it is said to be anticipated by Heinegård *et al.* (WO 98/46253).

Heinegård *et al.* (WO 98/46253) teach purification of rat cartilage matrix oligomeric protein and bovine cartilage matrix oligomeric protein. Compositions comprising the protein can be made to administer to a mammal to treat arthritic conditions in the mammal. Heinegård *et al.* do not teach the composition of Claim 42. A careful analysis of the passage pointed out by the Examiner (page 15, lines 1-6) leads to the conclusion that the described composition of Heinegård *et al.* requires at least two components, one selected from list A, the other selected from list B:

A. cartilage matrix oligomeric protein
fragments of cartilage matrix oligomeric protein
analogues of cartilage matrix oligomeric protein

B. collagene II
collagene IX
collagene XI
aggrecan
fragments of collagene II
fragments of collagene IX
fragments of collagene XI
fragments of aggrecan
analogues of collagene II
analogues of collagene IX
analogues of collagene XI
analogues of aggrecan

A careful analysis of Claim 42 leads to the conclusion that the described composition of Claim 42 requires at least three components, one selected from list C, the second one selected from list D, and the third (and optionally, fourth, fifth, etc. selected from list E):

C. cartilage matrix oligomeric protein

D. type I collagen gel (in a biological matrix)
type II collagen gel (in a biological matrix)

E. (all components below are in a biological matrix)
treated cartilage matrices
treated bone matrices
collagens
hyaluronan
fibrin gels
carbon filters
porous polylactic acid

The cited reference does not disclose any of the combinations of Claim 42. No combination of elements, one from list A, and one or more from list B, can match any of the combinations of elements of lists C, D and E. Each of the elements of the combination from lists C, D and E must be specifically disclosed as a combination in the specification of Heinegård *et al.* The use of the word "comprising" in Heinegård *et al.* (see page 15, lines 1-6) does not mean that the reference teaches a composition with further unspecified components added. Such a composition is not anticipating under 35 U.S.C. § 102(a), as every element of the claim must be specifically described in the cited reference.

CONCLUSION

The Examiner is requested to consider the above amendments and remarks. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

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Insulinlike Growth Factor 1– and 2–Augmented Collagen Gel Repair of Facial Osseous Defects

James S. Toung, BA; Roy C. Ogle, PhD; Raymond F. Morgan, DDS, MD; William H. Lindsey, MD

Background: Defects of the facial bone structure are common problems for the facial plastic surgeon. Native type 1 collagen gels (T1CGs) have been shown to mediate repair of facial critical-size defects in rat models.

Objective: To evaluate the efficacy of T1CG augmented with insulinlike growth factor (IGF) 1, IGF-2, and a combination of IGF-1 and IGF-2 on the repair of facial critical-size defects in a rodent model.

Methods: Twenty-four retired male breeder Sprague-Dawley rats were divided into 4 groups of 6 animals. Facial critical-size defects were created by removing the nasalis bones with a bone-cutting drill. Defects were treated with 300 µg of type 1 collagen gel (T1CG), T1CG augmented with 3 µg of IGF-1, T1CG augmented with 3 µg of IGF-2, or T1CG augmented with a combination of 3 µg of IGF-1 and 3 µg of IGF-2. After 30 days the animals were examined at necropsy with precise planimetry, histological analysis of new bone growth, and radiodensitometric analysis of bone thickness.

Results: Radiodensitometric measurements showed that IGF-2 augmentation resulted in greatest osseous healing, with measurements being statistically significant over those of all other groups ($P \leq .03$). Combination IGF-1 and IGF-2 had osseous healing that was intermediate between IGF-1 augmentation and IGF-2 augmentation alone, with measurements being statistically significant over those of unaugmented gels ($P < .001$) and IGF-1 augmentation ($P \leq .03$). Augmentation with IGF-1 resulted in healing that was significant over that of unaugmented gels ($P \leq .04$).

Conclusion: Collagen gels augmented with IGF significantly enhance the osteoconductive repair of nasal critical-size defects in a rodent model, with IGF-2 showing highest efficacy.

Arch Otolaryngol Head Neck Surg. 1999;125:451-455

OSSEOUS DEFECTS of the nasalis and other facial bones can result from many processes, including congenital malformations, trauma, neoplasms, and infections. Current techniques for the repair of such defects have major limitations or risks. Autogenous bone grafts using the iliac crest or calvaria as a donor site are widely used in reconstructive surgery; however, the amount of graft material that can be obtained is limited and such techniques have risks of donor site morbidity.[1] Cadaveric or allogenic bone is also available for grafts, but there may be the potential for transferring viral pathogens.[2] Demineralized bone does promote bone repair but has unpredictable resorption rates, which may be influenced by local vasculature, inflammation, and poor HLA match between host and donor.[3] Alloplastic materials such as Silastic and silicone can be displaced and can foster infections.

Critical-size defects (CSDs) are osseous defects that show less than 10% healing in 6 months or within the life of the animal.[4] With a CSD, bone regeneration would only take place in the presence of materials that are osteogenic. A CSD therefore provides a simple model for evaluating the osteogenic capabilities of materials and methods used in bone repair, without the intervening variables found in models that prevent osseous union by inhibiting the healing process. The rat nasal defect fulfills criteria to be used as a CSD.[5] Type 1 collagen gels (T1CGs) have previously been shown to mediate the total surface repair of osseous nasal CSDs in Sprague-Dawley rats.[6]

Insulinlike growth factors (IGFs) have been shown to promote proliferation and protein synthesis in osteoblasts and are believed to be the route by which growth hormone mediates the growth of bone. Thaller et al[7] demonstrated that supplying IGF-1 systemically through an

From the Departments of Otolaryngology–Head and Neck Surgery (Mr Toung and Dr Lindsey), Plastic Surgery (Drs Ogle and Morgan), and Neurosurgery and Cell Biology (Dr Ogle), University of Virginia Health Sciences Center, Charlottesville.

MATERIALS AND METHODS

Twenty-four 6-month-old, retired male breeder Sprague-Dawley rats (450-500 g) were randomly assigned to 4 groups of 6 rats to receive collagen (group 1), collagen plus IGF-1 (group 2), collagen plus IGF-2 (group 3), and collagen plus IGF-1 plus IGF-2 (group 4).

All underwent an operative procedure to produce a CSD as described previously.[5] Animals were anesthetized with pentobarbital sodium, 17 mg/kg, and 10 mg of ketamine hydrochloride. The hair over the nose and frontal skull was shaved and treated with a depilatory agent. The head was placed in a cephalostat and the skin was incised from the nasal tip to the supraorbital glabella. The periosteum was incised and separated from the underlying bone for lateral retraction. With the use of a bone-cutting burr with a handheld drill, the nasalis bones were removed and a rectangular defect measuring 5 × 15 mm was created. The floor of this defect was the deep side of the superior nasal mucosal membranes. A diamond burr was used to shape the defect to the precise measurements. The nasal cavity was not violated. Copious saline irrigation was used to remove bone dust and debris. After the defect was created and cleaned of debris, a collagen gel (with or without IGF augmentation) was placed into the defect. The periosteum was closed with a 6-0 nylon suture. The skin was closed with 4-0 polypropylene. After recovery from anesthesia, the animals were returned to the vivarium for routine housing.

Thirty days after surgery, the rats were killed by intracardiac lidocaine injection. The entire nasal dorsum of each rat was removed by using a cutting burr and was cleaned. The defects then underwent radiography (Faxitron Series model 43085N; Hewlett-Packard, Palo Alto, Calif) with an exposure time of 30 seconds at 30 kV (X-OMAT-AR film; Eastman Kodak Co, Rochester, NY). The developed x-ray films were then scanned into a computer by means of a densitometer (Molecular Dynamics Personal Densitometer; Molecular Dynamics, Sunnyvale, Calif), and the radiodensity of the defects was quantified with ImageQuant NT version 4.3 software (Molecular Dynamics). The defects were then treated with decalcifying solution (J. T. Baxter Inc, Deerfield, Ill) and embedded into paraffin for sectioning and hematoxylin-eosin staining to histologically evaluate new bone growth.

Rat tail collagen was prepared as previously described.[6,10] Rat tail collagen (2.8 mg/mL) in ice-cold 0.5-mol/L acetic acid was dialyzed against unbuffered Dulbecco modified Eagle medium and sterilized by addition of 0.1% chloroform during dialysis. To form gels, type 1 collagen (2.8 mg/mL) in 0.5-mol/L acetic acid was mixed with 5× concentrated Dulbecco modified Eagle medium in purified water and buffered to a final pH of approximately 7.5, as indicated by phenol red, with the use of sodium bicarbonate; the volume was adjusted to a final concentration of 1 mg/mL with deionized water before incubation. Type 1 collagen gels were cast from 300-μL volumes of cold 1 mg/mL into 15 × 5-mm rectangular forms. Gels containing human recombinant IGF-1 and/or IGF-2 were created by adding 3 μg of IGF-1 and/or IGF-2 (Upstate Biotechnology Inc, Lake Placid, NY) in 0.5-mol/L acetic acid before the sodium bicarbonate was added. The doses of IGFs were based on hypothetical local distribution of IGFs, assuming equal distribution through a 500-g rat, using doses equivalent to those showing skeletal effects when delivered by osmotic infusion pumps in studies by Thaller et al.[7]

osmotic infusion pump accelerated the repair of calvarial CSDs in rats.[7] It was also previously shown that 3-dimensional T1CGs augmented with IGF-1 result in significantly increased healing over that of unaugmented collagen gels ($P<.04$).[8] Fewer studies have been conducted on the effects of IGF-2; however, in vitro studies on bone cell cultures seem to indicate that IGF-2 and IGF-1 have the same efficacy in stimulating protein synthesis but that higher levels of IGF-2 are necessary to achieve the same effects, indicating that IGF-2 has less potency.[9] This study used a nasal CSD to determine the effect of 3-dimensional T1CGs augmented with IGF-2 and combination IGF-1 and IGF-2 on the repair of endochondral bone defects.

RESULTS

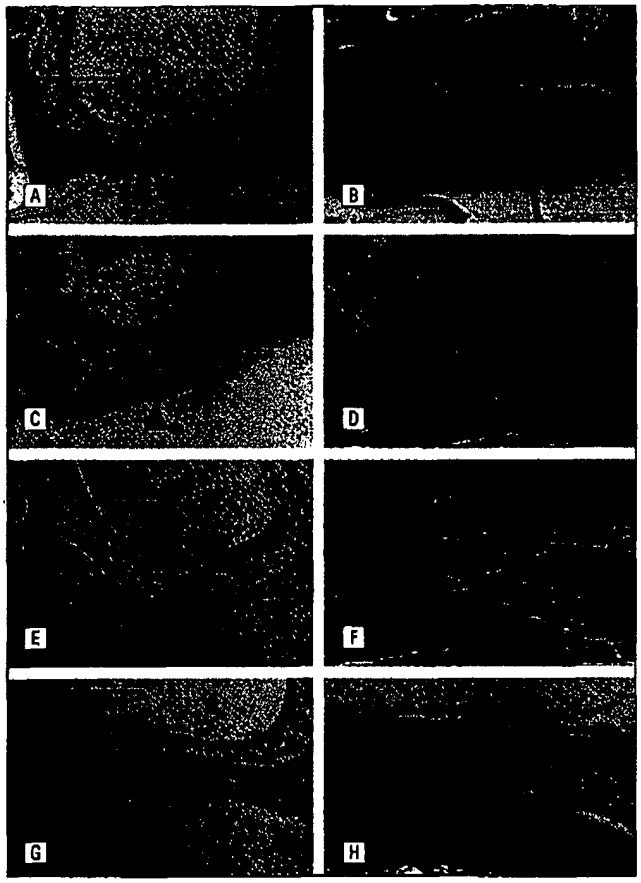
At necropsy, samples from all groups grossly appeared to have total surface healing, with the samples from the augmented groups appearing grossly thicker than samples of the unaugmented group. Bone densities and thicknesses of the excised nasal dorsums were compared by means of radiodensitometric values as explained previously.[8] Briefly, the samples were x-rayed and the developed films scanned into a computer. ImageQuant converts the radiopacity from the scanned x-ray image into arbitrary volume units, with greater radiopacity correlating with smaller volume units. Because these units of volume negatively correlate with radiopacity, we have designated these volume units as radiodensitometric values; therefore, smaller radiodensitometric values are obtained with greater bone healing.[8] Radiodensitometric values obtained from ImageQuant analysis of the x-ray films are presented in the **Table**. The mean (±SEM) for the collagen-only group was 5593.5 ± 61.9; the mean for the IGF-1 augmented group was 5175.0 ± 104.9. The mean for the IGF-2–augmented group was 4451.3 ± 87.3. The mean for the combination IGF-1 and IGF-2 group was 4826.2 ± 77.7. For comparison, a defect from a rat that was killed immediately after surgery yielded a value of 6128. A nasal bone harvested from a rat with no surgical defect yielded a value of 2880. Statistically significant differences were shown between paired groups by the Student *t* test.

Radiodensitometric measurements showed that the IGF-2 augmentation resulted in greatest osseous healing, with measurements being statistically significant over that of unaugmented gels ($P<.001$), IGF-1 augmentation ($P<.001$), and combination IGF-1 and IGF-2 augmentation ($P\leq.003$). Combination IGF-1 and IGF-2 had osseous healing that was intermediate between IGF-1 augmentation and IGF-2 augmentation alone, with measurements being statistically significant over those of unaugmented gels ($P<.001$) and IGF-1 augmentation

Radiodensitometric Values of X-rayed Nasal Defects*

No. Defect	No. Healing	Collagen	Collagen + IGF-1	Collagen + IGF-2	Collagen + IGF-1 and -2
2880	8128	5423	5287	4392	4678
		5528	5339	4198	4662
		5730	5363	4766	4896
		5741	4695	4275	4643
		5711	5071	4425	5079
		5428	5286	4652	4999
		5593.5 ± 61.9†	5175.0 ± 104.9†	4451.3 ± 87.3†	4826.2 ± 77.7†

*IGF indicates insulinlike growth factor.
†Mean ± SEM.



Defect treated with collagen augmented with combination insulinlike growth factor (IGF) 1 and IGF-2 at ×10 (A) and ×40 (B) magnification; collagen augmented with IGF-2 at ×10 (C) and ×40 (D) magnification; collagen only at ×10 (E) and ×40 (F) magnification; and collagen augmented with IGF-1 at ×10 (G) and ×40 (H) magnification (hematoxylin-eosin). Arrows represent the nasal septum; arrowheads, new bone growth.

(P≤.03). Augmentation with IGF-1 resulted in healing that was significant over that of unaugmented gels (P≤.04).

Histological examination of all groups showed complete surface coverage with a thin layer of immature bone. Visual comparison of bone thicknesses seen on histological examination (**Figure**) appeared to correlate with the radiodensitometric analysis (IGF-2>combination IGF-1 and IGF-2>IGF-1>collagen only). No evidence of inflammatory reactions was apparent histologically.

COMMENT

Augmentation of T1CG with IGF resulted in osseous healing that was significant over that of unaugmented collagen gels. Augmentation with IGF-2 resulted in greatest osseous healing, with measurements being statistically significant over those of unaugmented gels (P<.001), IGF-1 augmentation (P<.001), and combination IGF-1 and IGF-2 augmentation (P≤.003). Combination IGF-1 and IGF-2 had osseous healing that was intermediate between IGF-1 augmentation and IGF-2 augmentation alone, with measurements being statistically significant over those of unaugmented gels (P<.001) and IGF-1 augmentation (P≤.03). Augmentation with IGF-1 resulted in healing that was significant over that of unaugmented gels (P≤.04).

The technique of radiodensitometric analysis was used in this and 1 other previous study for estimating bone regeneration.[8] Other techniques were considered. Nuclear scanning for bone density is used for estimating large defect densities but is impractical for analyzing these small defect densities. Computed tomographic scans were used in our original study on type 1 collagen–mediated repair of nasal defects but were not used in this study because of limitations with defects less than 3 mm in depth.[6] The use of ultrasound in estimating bone density has been used by other investigators but is unavailable at our institution. In any study, analyzing bone density and regeneration is a potential weakness. We believe that this roentgenographic technique provides a good way to compare bone densities, particularly when used to compare radiodensities between flat bones such as the nasalis, which is more suitable for this technique than more complex-shaped bones. This method of analysis does not give a direct value of bone density, but the planar geometry of the excised dorsum does allow the 2-dimensional roentgenographic projections to be used for the comparison of healing between groups.

It has previously been shown that untreated nasal CSDs had less than 7% surface area healing during a 6-week time span, while repair with the use of unaugmented type 1 collagen resulted in 100% surface area healing.[6] Type 1 collagen is a major component of the extracellular matrix of bone. By laying down the 3-dimensional T1CG into the defect, the major component of the extracellular matrix is provided. A possible mechanism of increased healing, therefore, could be that the gel

provides a scaffold for osteoblast migration.[6] The addition of IGF-1 and IGF-2 confers additional osteoinductive properties and promotes healing of bone that is significantly greater than that of collagen gels alone. The use of IGF-1– and IGF-2–augmented T1CG offers advantages over currently used techniques in nasal reconstruction. Because the type 1 collagen used in the gels is part of the extracellular matrix of bone, it is readily incorporated into the healing defect and does not have the potential for extrusion at a later time. Many of the current techniques used in facial reconstruction possess notable risks of infection. This study and previous studies using T1CGs showed no signs of infection or inflammation.[6,10]

The increased healing with locally delivered IGF-1 and IGF-2 augmentation was not unexpected. Systemically released IGF-1 is believed to mediate the effects of growth hormone by stimulating synthesis of glycogen in the liver and the synthesis of collagen in bones.[11] During the development of children, growth hormone is believed to play a key role in the growth of long bone; the effects of growth hormone on bone growth are believed to be mediated by IGF-1 production.[12] Rats with calvarial CSDs that were administered IGF-1 with 14-day osmotic infusion pumps showed nearly complete healing after 6 weeks, while control rats showed nearly no healing after 8 weeks.[7] Both IGF-1 and IGF-2 have been shown to stimulate cell proliferation and collagen synthesis by osteoblasts as well as the synthesis of DNA, collagen, and noncollagen proteins in cultured rat calvaria.[11] In addition, IGF-1 and IGF-2 have been shown to decrease collagenase transcripts by more than 80% and thus decrease collagen breakdown.[13] While IGF-1 and IGF-2 are circulating hormones and can act systemically, local production of IGF-1 and IGF-2 by skeletal tissue is also an important source of these factors and may act as a paracrine or autocrine regulator of bone formation. In fact, it has been postulated that systemic circulating IGF-1 mediates some of its actions at the growth plates by stimulating the local production of IGF-1.[14]

The degrees to which IGF-1 and IGF-2 stimulate cell proliferation and collagen synthesis and suppress collagenase production in vitro are roughly equivalent, hence their in vitro efficacies appear to be comparable. Yet IGF-1 appears to be more potent than IGF-2, because it mediates these effects at lower molar concentrations. There are 2 known types of IGF receptors, type 1 and type 2, and both IGF-1 and IGF-2 interact with both types of receptors. In vitro evidence suggests that the stimulatory effects of both IGF-1 and IGF-2 on isolated bone and bone cell cultures are mediated by the type 1 receptors. Thus, it has been proposed that the lower biological potency displayed by IGF-2 in isolated bone and bone cell cultures results from the preference of the type 1 IGF receptor for binding IGF-1.[14]

In the rodent nasal CSD model, however, IGF-2–augmented collagen gels resulted in bone healing that was significantly greater than that of IGF-1–augmented collagen gels. This suggests that, in vivo, IGF-2 may have greater efficacy than IGF-1 in mediating the repair of osseous nasal defects in rats. Several possibilities may explain this surprising result. First, the effects of IGFs are strongly influenced by the balance of IGFs, IGF receptors, and IGF-binding proteins.[15] The in vitro and in vivo environments may cause cells to differ in the levels of expression of these proteins. Second, studies have shown that bones and bone cells taken from different parts of an organism have large differences in the local expression of IGFs, IGF-binding proteins, fibroblast growth factor, and alkaline phosphatase.[16,17] No study to date has compared cells from the nasalis bone with those of other sites. Third, IGF-1 and IGF-2 messenger RNA is normally preferentially expressed at different times during fracture repair and growth. For example, endothelial and mesenchymal cells at the granulation tissue stage express a predominance of IGF-2 messenger RNA. At the stage of bone and cartilage formation, osteoblasts and nonhypertrophic chondrocytes express messenger RNA for both IGF-1 and IGF-2. Osteoclasts are positive for IGF-2 messenger RNA at the stage of bone remodeling. In all stages, there is a predominance of IGF-2 in the human bone matrix.[18]

Most surprising of all was that augmentation of the gels with a combination of both IGF-1 and IGF-2 resulted in healing that was intermediate between that of IGF-1 augmentation and IGF-2 augmentation alone. There was twice as much total IGF present in these gels (6 µg total). The observed healing suggests that some inhibition of IGF-2 by IGF-1 might be explained if both factors act through the same receptors, as the 2 factors would compete for the same receptor binding sites. In vitro evidence does suggest that both IGFs interact at the same receptors but with different affinities. Another possible explanation is a biphasic response. With this type of response, increasing concentrations of the growth factor may increase growth at low concentrations, but, with greater concentrations, decreased healing may result. This type of dose-response activity is seen with transforming growth factor-β.[19]

An advantage of using IGF-augmented collagen gels over systemic delivery of IGFs is that the growth factors are directed to the site of the defect. This provides a more controlled dosage to the site, reduces the amount of IGFs needed, and reduces the effects of high doses of systemic IGFs. Unlike the cranial vault, the nasalis bone forms by endochondral ossification rather than intramembranous ossification. This is the first study, to our knowledge, to look at the effects of a known concentration of IGF-2 delivered locally to a CSD in a site formed by endochondral ossification. The techniques to evaluate IGF-2 in higher animals exist, and the materials are currently available for such studies. The use of IGF-2 is approved in humans, and techniques for obtaining human collagen from amnions have been developed.[20]

CONCLUSIONS

Rat nasal defects treated with IGF-2–augmented T1CGs showed healing that was significantly greater than that of collagen only, IGF-1 augmentation, and combination IGF-1 and IGF-2 augmentation. Rat nasal defects treated with combination IGF-1– and IGF-2–augmented T1CGs showed healing that was intermediate between that of IGF-1 augmentation alone and IGF-2 augmentation alone, being significant over that of IGF-1 augmentation alone and collagen-only treatment. Rat nasal defects treated with IGF-

1–augmented collagen gels showed healing that was significant over that in rats treated with nonaugmented collagen gels. The use of IGF-augmented collagen gels in the repair of nasal defects merits further investigation.

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MOLECULAR BIOLOGY OF THE CELL

fourth edition

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Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

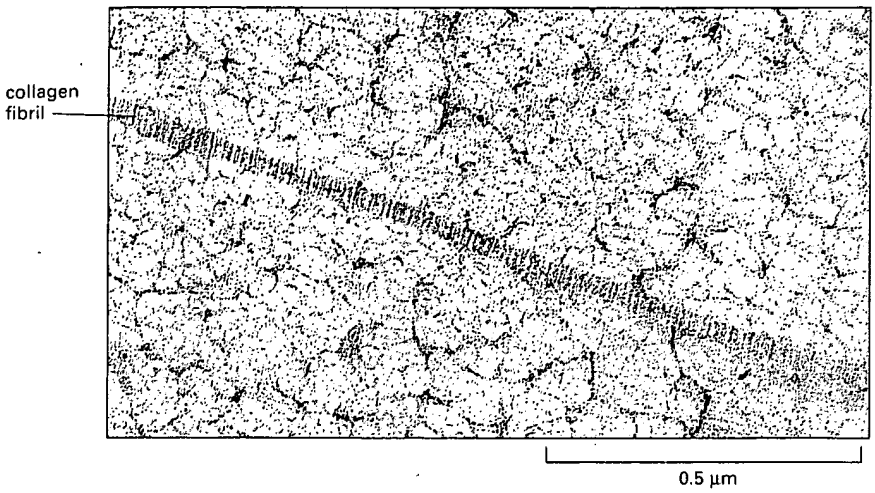

Figure 19–42 Proteoglycans in the extracellular matrix of rat cartilage. The tissue was rapidly frozen at −196°C, and fixed and stained while still frozen (a process called freeze substitution) to prevent the GAG chains from collapsing. In this electron micrograph, the proteoglycan molecules are seen to form a fine filamentous network in which a single striated collagen fibril is embedded. The more darkly stained parts of the proteoglycan molecules are the core proteins; the faintly stained threads are the GAG chains. (Reproduced from E.B. Hunziker and R.K. Schenk, *J. Cell Biol.* 98:277–282, 1985. © The Rockefeller University Press.)

the response of cells to some extracellular signal proteins. In addition, some conventional receptors have one or more GAG chains and are therefore proteoglycans themselves.

Among the best-characterized plasma membrane proteoglycans are the *syndecans,* which have a membrane-spanning core protein. The extracellular domains of these transmembrane proteoglycans carry up to three chondroitin sulfate and heparan sulfate GAG chains, while their intracellular domains are thought to interact with the actin cytoskeleton in the cell cortex.

Syndecans are located on the surface of many types of cells, including fibroblasts and epithelial cells, where they serve as receptors for matrix proteins. In fibroblasts, syndecans can be found in focal adhesions, where they modulate integrin function by interacting with fibronectin on the cell surface and with cytoskeletal and signaling proteins inside the cell. Syndecans also bind FGFs and present them to FGF receptor proteins on the same cell. Similarly, another plasma membrane proteoglycan, called *betaglycan,* binds TGF-β and may present it to TGF-β receptors.

The importance of proteoglycans as co-receptors is illustrated by the severe developmental defects that can occur when specific proteoglycans are inactivated by mutation. In *Drosophila,* for example, signaling by the secreted signal protein *Wingless* depends on the protein's binding to a specific heparan sulfate proteoglycan co-receptor called *Dally* on the target cell. In mutant flies deficient in Dally, Wingless signaling fails, and the severe developmental defects that result are similar to those that result from mutations in the *wingless* gene itself. In some tissues, inactivation of Dally also inhibits signaling by a secreted protein of the TGF-β family called *Decapentaplegic (DPP).*

Some of the proteoglycans discussed in this chapter are summarized in Table 19–4.

Collagens Are the Major Proteins of the Extracellular Matrix

The **collagens** are a family of fibrous proteins found in all multicellular animals. They are secreted by connective tissue cells, as well as by a variety of other cell types. As a major component of skin and bone, they are the most abundant proteins in mammals, constituting 25% of the total protein mass in these animals.

The primary feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called *α chains,* are wound around one another in a ropelike superhelix (Figure 19–43). Collagens are extremely rich in proline and glycine, both of which are important in the formation of the triple-stranded helix. Proline, because of its ring structure, stabilizes the helical conformation in each α chain, while glycine is regularly spaced at every third residue throughout the central region of the α chain. Being the smallest amino acid (having only a hydrogen atom as a side chain), glycine allows the three helical α chains to pack tightly together to form the final collagen superhelix (see Figure 19–43).

TABLE 19–4 Some Common Proteoglycans

PROTEOGLYCAN	APPROXIMATE MOLECULAR WEIGHT OF CORE PROTEIN	TYPE OF GAG CHAINS	NUMBER OF GAG CHAINS	LOCATION	FUNCTIONS
Aggrecan	210,000	chondroitin sulfate + keratan sulfate	~130	cartilage	mechanical support; forms large aggregates with hyaluronan
Betaglycan	36,000	chondroitin sulfate/ dermatan sulfate	1	cell surface and matrix	binds TGF-β
Decorin	40,000	chondroitin sulfate/ dermatan sulfate	1	widespread in connective tissues	binds to type I collagen fibrils and TGF-β
Perlecan	600,000	heparan sulfate	2–15	basal laminae	structural and filtering function in basal lamina
Syndecan-1	32,000	chondroitin sulfate + heparan sulfate	1–3	epithelial cell surface	cell adhesion; binds FGF and other growth factors
Dally (in *Drosophila*)	60,000	heparan sulfate	1–3	cell surface	co-receptor for Wingless and Decapentaplegic signaling proteins

So far, about 25 distinct collagen α chains have been identified, each encoded by a separate gene. Different combinations of these genes are expressed in different tissues. Although in principle more than 10,000 types of triple-stranded collagen molecules could be assembled from various combinations of the 25 or so α chains, only about 20 types of collagen molecules have been found. The main types of collagen found in connective tissues are types I, II, III, V, and XI, type I being the principal collagen of skin and bone and by far the most common. These are the **fibrillar collagens**, or fibril-forming collagens, with the ropelike structure illustrated in Figure 19–43. After being secreted into the extracellular space, these collagen molecules assemble into higher-order polymers called *collagen fibrils*, which are thin structures (10–300 nm in diameter) many hundreds of micrometers long in mature tissues and clearly visible in electron micrographs (Figure 19–44; see also Figure 19–42). Collagen fibrils often aggregate into larger, cablelike bundles, several micrometers in diameter, which can be seen in the light microscope as *collagen fibers*.

Collagen types IX and XII are called *fibril-associated collagens* because they decorate the surface of collagen fibrils. They are thought to link these fibrils to one another and to other components in the extracellular matrix. Types IV and VII are *network-forming collagens*. Type IV molecules assemble into a feltlike sheet or meshwork that constitutes a major part of mature basal laminae, while type VII molecules form dimers that assemble into specialized structures called *anchoring fibrils*. Anchoring fibrils help attach the basal lamina of multilayered epithelia to the underlying connective tissue and therefore are especially abundant in the skin.

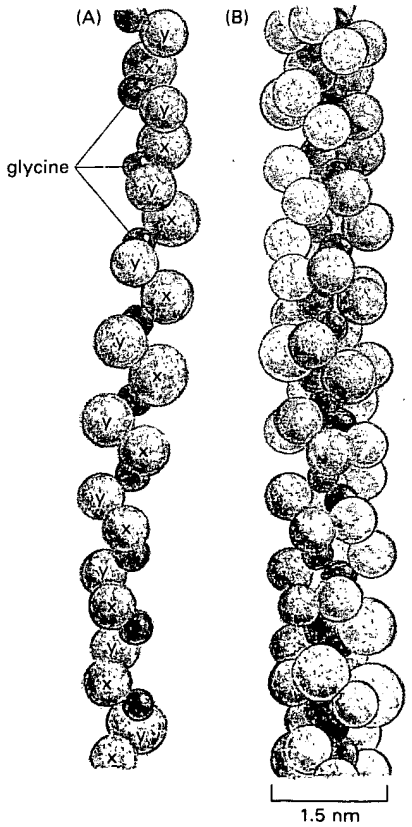

Figure 19–43 The structure of a typical collagen molecule. (A) A model of part of a single collagen α chain in which each amino acid is represented by a sphere. The chain is about 1000 amino acids long. It is arranged as a left-handed helix, with three amino acids per turn and with glycine as every third amino acid. Therefore, an α chain is composed of a series of triplet Gly-X-Y sequences, in which X and Y can be any amino acid (although X is commonly proline and Y is commonly hydroxyproline). (B) A model of part of a collagen molecule in which three α chains, each shown in a different color, are wrapped around one another to form a triple-stranded helical rod. Glycine is the only amino acid small enough to occupy the crowded interior of the triple helix. Only a short length of the molecule is shown; the entire molecule is 300 nm long. (From model by B.L. Trus.)

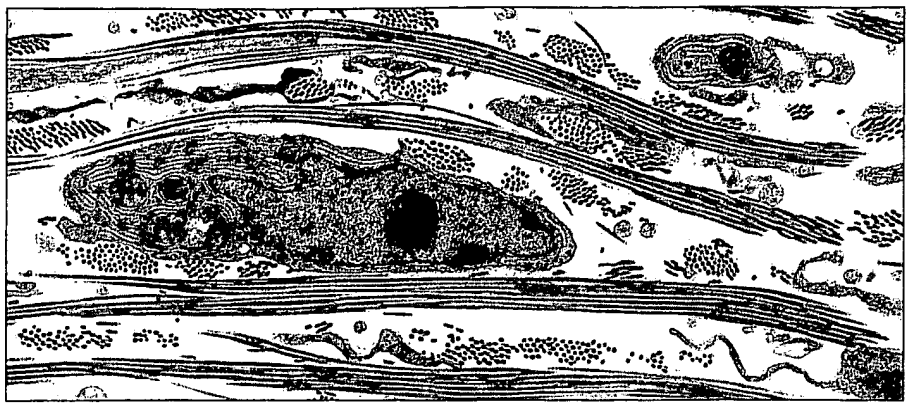

Figure 19–44 Fibroblast surrounded by collagen fibrils in the connective tissue of embryonic chick skin. In this electron micrograph, the fibrils are organized into bundles that run approximately at right angles to one another. Therefore, some bundles are oriented longitudinally, whereas others are seen in cross section. The collagen fibrils are produced by the fibroblasts, which contain abundant endoplasmic reticulum, where secreted proteins such as collagen are synthesized. (From C. Ploetz, E.I. Zycband, and D.E. Birk, *J. Struct. Biol.* 106:73–81, 1991. © Academic Press.)

There are also a number of "collagen-like" proteins, including type XVII, which has a transmembrane domain and is found in hemidesmosomes, and type XVIII, which is located in the basal laminae of blood vessels. Cleavage of the C-terminal domain of type XVIII collagen yields a peptide called *endostatin,* which inhibits new blood vessel formation and is therefore being investigated as an anticancer drug. Some of the collagen types discussed in this chapter are listed in Table 19–5.

Many proteins that contain a repeated pattern of amino acids have evolved by duplications of DNA sequences. The fibrillar collagens apparently arose in this way. Thus, the genes that encode the α chains of most of these collagens are very large (up to 44 kilobases in length) and contain about 50 exons. Most of the exons are 54, or multiples of 54, nucleotides long, suggesting that these collagens arose by multiple duplications of a primordial gene containing 54 nucleotides and encoding exactly 6 Gly-X-Y repeats (see Figure 19–43).

Collagens Are Secreted with a Nonhelical Extension at Each End

Individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and injected into the lumen of the endoplasmic reticulum (ER) as

TABLE 19–5 Some Types of Collagen and Their Properties

	TYPE	MOLECULAR FORMULA	POLYMERIZED FORM	TISSUE DISTRIBUTION
Fibril-forming (fibrillar)	I	$[a1(I)]_2\alpha2$ (I)	fibril	bone, skin, tendons, ligaments, cornea, internal organs (accounts for 90% of body collagen)
	II	$[\alpha1(II)]_3$	fibril	cartilage, invertebral disc, notochord, vitreous humor of the eye
	III	$[\alpha1(III)]_3$	fibril	skin, blood vessels, internal organs
	V	$[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$	fibril (with type I)	as for type I
	XI	$\alpha1(XI)\alpha2(IX)\alpha3(XI)$	fibril (with type II)	as for type II
Fibril-associated	IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	lateral association with type II fibrils	cartilage
	XII	$[\alpha1(XII)]_3$	lateral association with some type I fibrils	tendons, ligaments, some other tissues
Network-forming	IV	$[\alpha1(IV)]_2\alpha2(IV)$	sheetlike network	basal lamina
	VII	$[\alpha1(VII)]_3$	anchoring fibrils	beneath stratified squamous epithelia
Transmembrane	XVII	$[\alpha1(XVII)]_3$	not known	hemidesmosomes
Others	XVIII	$[\alpha1(XVIII)]_3$	not known	basal lamina around blood vessels

Note that types I, IV, V, IX, and XI are each composed of two or three types of α chains, whereas types II, III, VII, XII, XVII, and XVIII are composed of only one type of α chain each. Only 11 types of collagen are shown, but about 20 types of collagen and about 25 types of α chains have been identified so far.